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Farnesyl Thiosalicylic Acid Chemosensitizes Human Melanoma *In Vivo*

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Malignant melanoma is well known for its poor response to a variety of chemotherapeutic agents. Testing of numerous treatment strategies has identified dacarbazine as the most active single drug; however, its response rates in various clinical settings are quite limited. Defective apoptosis in combination with oncogenic proteins (such as activated Ras) in cell proliferation pathways plays a key part in both the development and disease progression of human melanoma. Farnesyl thiosalicylic acid, a novel Ras inhibitor, dislodges Ras proteins from the cell membrane, leading to inhibition of cell transformation and tumor growth. In this study we evaluated the effect of farnesyl thiosalicylic acid treatment on established human melanoma xenografts grown in mice with severe combined immunodeficiency as well as the chemosensitizing effect of farnesyl thiosalicylic acid in combination with dacarbazine. Daily administration of 10, 20, or 40 mg per kg of farnesyl thiosalicylic acid resulted in a concentration-dependent reduction in tumor growth, with growth inhibition reaching a mean value of 45±7%, at the highest concentration. The combination of farnesyl thiosalicylic acid (10 mg per kg per day) and dacarbazine (80 mg per kg per day) resulted in a significant reduction of 56%±9%, in mean tumor growth. Analysis of toxicologic parameters (mouse weight, blood cell counts, and blood chemistry) showed an acceptable and similar toxicity profile for both the single-agent farnesyl thiosalicylic acid treatment and the combination of farnesyl thiosalicylic acid plus dacarbazine treatment. Given the observed preclinical treatment responses and the low toxicity, our results support the notion that farnesyl thiosalicylic acid in combination with dacarbazine may qualify as a rational treatment approach for human melanoma. ***Key words: dacarbazine/farnesyl thiosalicylic acid/severe combined immunodeficiency/toxicity. J Invest Dermatol 120:000–000, 2003***

Malignant melanoma is one of the most rapidly increasing malignancies in the white population and has a mortality rate surpassed only by lung cancer (Slominski *et al*, 2001). Age-adjusted incidence rates are 12 per 100,000 in the U.S.A. and about three times higher in some geographic areas, such as Australia (Helmbach *et al*, 2001). Malignant melanoma is a tumor derived from activated or genetically altered melanocytes as a result of complex interactions between genetic, constitutional, and environmental factors (Slominski *et al*, 1998, 2001; Halpern and Altman, 1999). Early, localized melanoma has a good prognosis after adequate surgical therapy. The prognosis of metastatic melanoma, however, is poor despite the availability of a growing number of biologic, chemotherapeutic, and combined modality treatments. Today, dacarbazine is the most widely used single-agent chemotherapy for metastatic melanoma, but few patients obtain durable responses, and the long-term survival benefit from this drug is limited (Middleton *et al*, 2000) due to the exceptionally high degree of drug resistance of this malignancy (Helmbach *et al*, 2001). Dacarbazine, a methylating agent, requires metabolic activation in the liver through an N-demethylation reaction by the CYP450 system, and subsequently undergoes spontaneous cleavage to yield a stable metabolite (AIAC) and an active methylating species, diazomethane (Chabner *et al*, 1996; Reid *et al*, 1999). Therefore, dacarbazine has no *in vitro* activity and is suitable only in *in vivo* models. In an attempt to improve chemotherapy response rates in malignant melanoma, numerous clinical trials have been performed using chemotherapy (with or without inclusion of dacarbazine) as well as immunotherapy (with interferons±interleukin-2), gene therapy, or combinatorial strategies. Unfortunately, it remains unclear whether any of these combination therapies is superior to single-agent dacarbazine in this disease (reviewed in Huncharek *et al*, 2001). Thus, new treatment approaches are still being sought.

Although a multitude of factors have been suspected of participating in melanoma growth, progression, and chemoresistance, the most common specific gene defects identified in melanoma are activating mutations in Ras genes (Van't Veer *et al*, 1989; Ball *et al*, 1994). The incidence of Ras gene mutations in human melanoma is about 20% (Ball *et al*, 1994; van-Elsas *et al*, 1996), representing mainly alterations in N-Ras at codon 61, whereas alterations in H-Ras and Ki-Ras are relatively rare (Van't Veer *et al*, 1989; Ball *et al*, 1994; van-Elsas *et al*, 1995). Because Ras proteins

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Abbreviations: FTS, S-*trans, trans* farnesylthiosalicylic acid; FTI, farnesyl transferase inhibitors; SCID, severe combined immunodeficiency.

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are regulators of multiple signaling pathways that control cell growth, differentiation, and apoptosis (Boguski and McCormick, 1993; Marshall, 1996; Bos, 1997), the deregulation of other cellular factors may also mimic effects of aberrant Ras function even in the absence of a Ras mutation. Thus, the influence of normal and aberrant Ras function on the biology of human melanoma may be much greater than expected from the frequency of Ras gene mutations in this tumor. This notion further highlights the potential benefits that Ras inhibitors may provide in attempts to block the growth of malignant melanoma.

Improved understanding of the molecular mechanisms of Ras processing and membrane targeting provided an important background for the development of compounds that would inhibit the association of Ras with the inner cell membrane. Such compounds include farnesyltransferase inhibitors (FTI), as well as inhibitors of the prenyl-CAAX protease, inhibitors of the methyltransferase, possible inhibitors of Ras trafficking, and inhibitors such as S-*trans*, *trans* farnesyl thiosalicylic acid (FTS) that interfere with the association of mature Ras with the cell membrane. FTI, the most widely studied group of Ras inhibitors, fail to inhibit the functions of N-Ras and K-Ras proteins, as both of these isoforms can be geranyl-geranylated under conditions where the farnesyl transferase is inhibited. These alternatively prenylated Ras isoforms are still targeted to the cell membrane and remain active (Kloog *et al*, 1999).

The farnesyl group, which is common to all Ras proteins, may act as part of a recognition unit for specific anchorage lipids or protein(s) that interact with Ras in the cell membrane (Cox and Der, 1997). On the assumption that Ras functions might be inhibited by competitively displacing the mature protein from its putative membrane-anchorage domains, a series of organic compounds resembling the farnesylcysteine of Ras proteins was designed (Marciano *et al*, 1995; Aharonson *et al*, 1998). One of these compounds, FTS, was found to be a potent growth inhibitor of H-Ras-transformed Rat-1 (EJ) fibroblasts, specifically affecting the membrane-bound H-Ras protein in these cells (Marom *et al*, 1995; Haklai *et al*, 1998). Specificity of FTS towards Ras was suggested by the finding that FTS can inhibit the growth of fibroblasts transformed by ErbB2 acting upstream of Ras, but not the growth of cells transformed by v-Raf (which, unlike Raf-1, acts independently of Ras). FTS does not inhibit the farnesylation of H-Ras; it affects H-Ras–membrane interactions both *in vitro* and *in vivo*, dislodging the protein from its anchorage domains, thus facilitating its degradation and hence reducing the total amount of cellular Ras (Marom *et al*, 1995; Haklai *et al*, 1998).

On the basis of these proposed general mechanisms, the therapeutic potential of FTS was tested *in vitro* in several cell lines of different origin. FTS was found to dislodge Ras proteins in all cells investigated so far, including H-Ras-, K-Ras-, and N-Ras-transformed rat fibroblasts (Marom *et al*, 1995; Haklai *et al*, 1998; Jansen *et al*, 1999), Rat-1 cells (Haklai *et al*, 1998), PC-12 cells (Haring *et al*, 1998), Panc-1 cells (Weisz *et al*, 1999), and colon carcinoma cells (Halaschek-Wiener *et al*, 2000). Furthermore, recent experiments showed that FTS reduces the amounts of activated N-Ras and wild-type Ras isoforms in both human melanoma cells and Rat-1 fibroblasts, blocks the activity of the Ras-dependent extracellular signal-regulated kinase in melanoma cells, and inhibits the growth of N-Ras-transformed fibroblasts and human melanoma cells *in vitro* and reverses their transformed phenotype. FTS also inhibits the growth of human melanoma cells injected into mice with severe combined immunodeficiency (SCID) without evidence of drug-related toxicity (Jansen *et al*, 1999).

In addition to its Ras-dislodging property, FTS upregulates the tumor suppressor protein p53 in colon cancer cells, leading to cell cycle arrest in G_0/G_1 via the $p53/p21^{(wafl/cipl)}$ signaling pathway (Halaschek-Wiener *et al*, 2000). Taken together, these findings support the notion that a change in the Ras-signaling cascade leads to complex restructuring of the general survival strategies of human cancer cells; however, H-Ras-transformed Rat-1 fibroblasts, Panc-1, and SW480 cells exposed to high concentrations of the Ras inhibitor FTS for prolonged periods (Gana-Weisz *et al*, 2002) did not establish resistance against the drug. Furthermore, it was shown that both Panc-1 and SW480 cancer cells were highly chemosensitized to gemcitabine and doxorubicin *in vitro*. Chemosensitization of Panc-1 tumors by FTS was also demonstrated in nude mice, with a synergistic effect on the survival rate of the combined treatment of gemcitabine and FTS (Gana-Weisz *et al*, 2002).

On the basis of these observations, it seemed reasonable to speculate that FTS might chemosensitize human melanoma *in vivo*. In this study we used FTS in a dacarbazine-based combination chemotherapy in a human melanoma SCID mouse xenotransplantation model. We evaluated the effects of both single-agent treatment with FTS or dacarbazine and combinations of FTS and dacarbazine on established melanoma xenografts. FTS inhibited tumor growth in a concentration-dependent manner and chemosensitized melanomas harboring an activated N-Ras gene. Analysis of toxicologic parameters yielded a highly favorable toxicity profile for both single-agent FTS and combined treatment with FTS and dacarbazine.

MATERIALS AND METHODS

Materials Clinical grade FTS and CRYSMEB (cyclodextrin) were obtained from Thyreos Corporation (Newark, NJ). Dacarbazine was from MEDAC (Hamburg, Germany). FTS was formulated in CRYSMEB, according to the manufacturer's instructions, to increase its solubility by covering the hydrophobic farnesyl moiety. The stock solution of phosphate-buffered saline (PBS; Gibco, Carlsbad, CA) containing FTS in CRYSMEB (10% wt/wt) was stable for the duration of the experiments. CRYSMEB in PBS served as a control ("carrier control") in all experiments. Dacarbazine was dissolved in sterile H_2O to a final concentration of 10 mg per ml and the required amount of dacarbazine was injected intraperitoneally.

Cell culture The human melanoma cell line 607B was obtained from Dr Peter Schrier, Leiden, the Netherlands. These cells harbor a naturally occurring N-Ras gene mutation (van-Elsas *et al*, 1995). Cells were cultured in Dulbecco minimal Eagle's medium (Gibco) supplemented with 10% fetal calf serum (Gibco) in a humidified 5% CO_2/95% ambient air atmosphere at 37°C. The cells needed for the experiments were grown in culture flasks, harvested on the day of injection, counted, and diluted in sterile PBS to a final concentration of 1×10^7 cells per 200 μl.

Experimental animals, tumor generation, and therapy Pathogen-free female C.B-17 scid/scid (SCID) mice, 4–6 wk old, tested for leakiness, were obtained from Harlan-Winkelmann (Borchen, Germany). Mice were housed in laminar flow racks and microisolator cages under specific pathogen-free conditions and received autoclaved food and water. SCID mice were injected subcutaneously (s.c.) into the left lower flank with 1×10^7 (200 μl) human melanoma cells (607B). One week after cell inoculation, when palpable tumors were established (0.5–0.7 mm^3), mice were randomized into the respective groups ($n = 8$ in each group) and treatment was initiated. FTS (treatment), carrier solution (CRYSMEB), or physiologic NaCl solution (saline control) was administered daily for 4 wk via the intraperitoneal (i.p.) route at 10, 20, or 40 mg FTS per kg. In a combination therapy experiment FTS (10 mg per kg) was injected i.p. daily for 2 wk, and dacarbazine (80 mg per kg) was injected i.p. on 5 consecutive days starting on day 1 of FTS treatment. This treatment regimen was followed by a 2 wk observation period. Tumor size was measured twice a week during treatment and during the observation period. Tumor volumes were calculated as follows: $V\ (mm^3) = (\text{length} \times \text{width}^2) \times 0.5$. Four weeks after the start of treatment the mice were killed, the tumors were isolated, and body and tumor weights were recorded. The results (tumor weights) are presented as the mean tumor weights (mean ± SD) of eight individual mice. Whole blood for blood cell analysis was withdrawn from mice in the combination chemotherapy study. All animal studies were approved by the University of Vienna Animal Welfare Committee.

Statistical significance Statistical significance of differences in mean tumor weights (in FTS-treated or combination-treated mice compared with controls) was determined using the Mann–Whitney U-test. Multiple comparisons were calculated using one-way ANOVA, and

post-tests among treatment groups were carried out using the Scheffé test (SPSS, release 10.0.7, Chicago, IL). $p < 0.05$ was considered to be statistically significant.

Toxicologic evaluation of FTS/dacarbazine combination therapy Tumor-free mice of the same type as the experimental mice were used to investigate the toxicity profile of FTS (20 mg per kg) with and without dacarbazine treatment (80 mg per kg). Mice treated with single-agent FTS or single-agent dacarbazine ($n = 8$ per group) were injected with the respective drugs for 3 d and were killed on day 4. Mice receiving the drugs in combination were treated for 4 d. Mice that received FTS plus dacarbazine combination treatment ($n = 8$ per day) were killed on days 3, 4, and 5 after the initial drug application. Their bodies were weighed and whole blood for blood cell analysis ($n = 4$) and serum for enzyme activity assays ($n = 4$) was obtained.

Blood cells and serum analysis For analysis of blood cells, 0.5–0.8 ml of whole blood was withdrawn ($n = 4$ from each group) in syringes rinsed with heparin (Baxter, Deerfield, IL). Concentrations of white and red blood cells and platelets were determined by fluorescence-activated cell sorter analysis (FACStar, Becton-Dickinson, San Jose, CA). Serum was isolated by centrifugation at 800 g. in an Eppendorf centrifuge at 4°C after incubation for 2 h at 4°C, and analyzed for liver, pancreas, and kidney function by standard laboratory procedures.

RESULTS

The main purpose of this study was to evaluate the therapeutic potential and the toxicity of single-agent FTS treatment as well as its chemosensitizing effect when administered in combination with dacarbazine. First we evaluated the therapeutic effect of FTS on established xenografts grown subcutaneously. In all the xenografted mice, xenotransplanted 607B cells grew as localized tumors and no evidence of metastasis was observed. We were unable to detect any metastasizing 607B melanoma cells in the main organs removed from the mice at the end of the experiments (data not shown).

Single-agent FTS treatment was initiated 1 wk after cell inoculation, when small tumors (0.5–0.7 mm^3) were palpable. Experimental animals were treated with 10, 20, or 40 mg FTS per kg or with the carrier (CRYSMEB) or saline (physiologic NaCl) control solutions (**Fig 1**). After 4 wk of daily treatment, the mice were killed and the mean tumor weights ($n = 8$ in each group) were recorded. As shown in **Fig 1(*A*)**, we observed a concentration-dependent decrease in tumor size. Carrier control treatment had only a small effect on tumor growth (the mean value was $-11\% \pm 2\%$); the FTS-treated groups showed concentration-dependent growth reduction of $31\% \pm 5\%$, $38\% \pm 8\%$, and $45\% \pm 7\%$ at 10, 20, and 40 mg FTS per kg, respectively, relative to saline-treated controls. FTS treatment at 40 mg per kg resulted in significant growth inhibition compared with both the carrier ($p < 0.01$) and saline ($p < 0.001$) control groups, whereas mice in the groups treated with 10 or 20 mg FTS per kg showed significant reductions in xenograft growth compared with the saline control ($p < 0.01$) but not to the carrier control group. Actual mean tumor weights ($n = 8$ mice in each group) were 1.9 g ± 0.17 g, for the saline controls, 1.7 g ± 0.56 g for the carrier controls, and 1.3 g ± 0.22 g, 1.2 g ± 0.19 g, and 1.0 g ± 0.16 g for the groups treated with FTS at 10, 20, and 40 mg per kg, respectively (**Fig 1*A***). These results clearly demonstrate concentration-dependent inhibition of mean tumor growth by FTS. Monitoring of body weight as an indicator of toxic side-effects revealed a nonsignificant reduction in mean body weight at the highest dose used ($-14\% \pm 2\%$ in the 40 mg FTS per kg group) (**Fig 2*A***). FTS reduced tumor growth in a time- and concentration-dependent manner (**Fig 1*B***): mice treated with FTS at 40 mg per kg clearly displayed the smallest xenografts, a finding that was noticeable as early as day 11. Only the xenografts of mice treated with FTS at 20 mg per kg were of similar size until day 21 (**Fig 1*B***).

In view of these findings, we carried out follow-up experiments combining dacarbazine chemotherapy with FTS to evaluate the chemosensitization potential of the Ras antagonist. In the first experiment, single-agent treatment with FTS at 10 mg per kg per day led to a small reduction in tumor growth, which was statistically nonsignificant compared with the carrier control. A

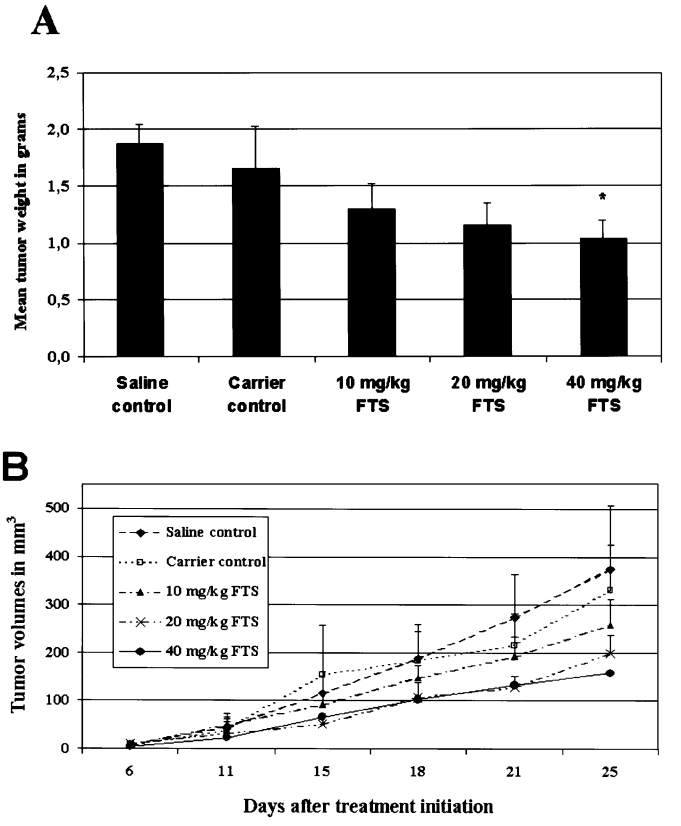

Figure 1. Inhibition of human melanoma growth in SCID mice by FTS. Mice were injected subcutaneously in the lower left flank with 1×10^7 607B human melanoma cells and were treated with FTS (10, 20, or 40 mg per kg, i.p., daily) for 4 wk. Treatment was initiated 1 wk after cell inoculation, when palpable tumors were established. Control mice were similarly injected with the carrier solution (carrier control) or physiologic NaCl solution (saline control). (*A*) Tumor weights (mean ± SD, $n = 8$) after 4 wk of FTS administration are presented. (*B*) Time- and concentration-dependent growth inhibition was monitored by caliper measurements of tumor volumes over the whole treatment period. Tumor volumes (mean ± SD, $n = 8$) are shown. An asterisk (*) indicates statistical significance compared with the control groups (carrier and saline), with $p < 0.01$.

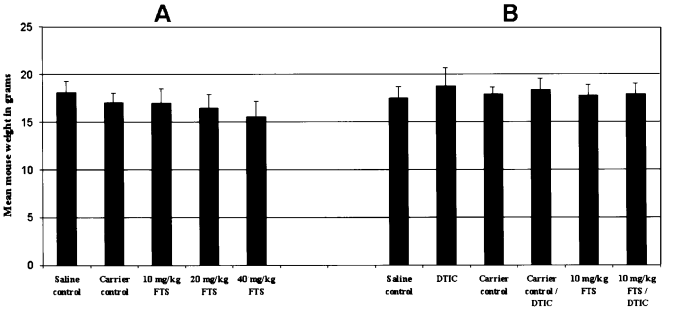

Figure 2. Body weight assessment as a toxicologic indicator. Mouse body weight (mean ± SD, $n = 8$) of mice was recorded at the end of single-agent FTS treatment (*A*) or combination treatment (*B*) experiments to determine the toxic effects of these treatment regimens. Neither the FTS treatment shown in *A* nor the treatments (dacarbazine alone or in combination with FTS) shown in *B* yielded a significant toxicologic effect as indicated by altered body weights.

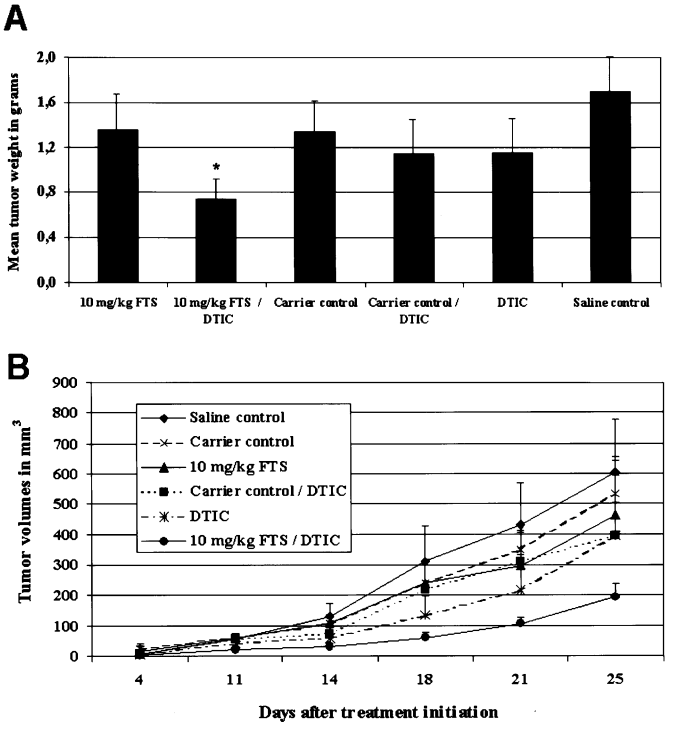

Figure 3. FTS chemosensitizes human melanoma in a SCID mouse xenotransplantation model. Mice were injected subcutaneously in the lower left flank with 1×10^7 607B human melanoma cells and then treated with FTS alone (10 mg per kg, i.p., daily for 2 wk) or dacarbazine alone (80 mg per kg, i.p., for 5 consecutive days) or a combination of FTS and dacarbazine. Treatment was started 1 wk after cell inoculation, when palpable tumors were established. Control mice were injected with the carrier solution (carrier control), a combination of the carrier and dacarbazine, or physiologic NaCl solution (saline control). (*A*) Tumor weights (mean $\pm$ SD, n = 8) 4 wk after treatment initiation (i.e., after 2 wk of drug administration and 2 wk of observation) are shown. (*B*) Tumor growth was monitored by caliper measurement of tumor volumes during the treatment and observation periods. Tumor volumes (mean $\pm$ SD, n = 8) are shown. An asterisk (*) indicates statistical significance compared with all other groups, with $p < 0.008$.

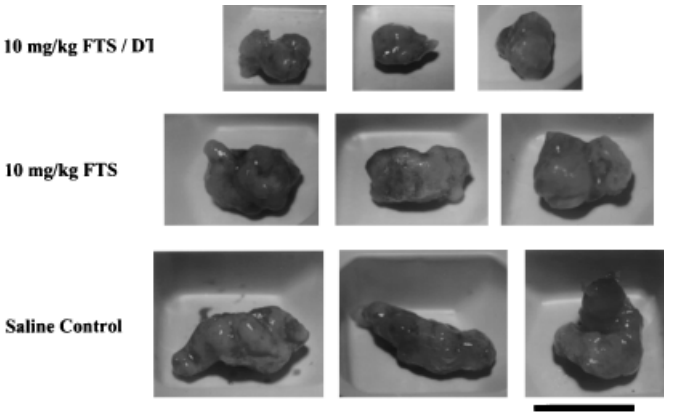

Figure 4. Photographs of excised tumors. At the end of the combination therapy experiment the mice were killed, the tumors were removed, and representative xenografts were photographed. Three representative tumors from the saline control group, the group treated with 10 mg FTS per kg, and the combination treatment group (10 mg FTS per kg and 80 mg dacarbazine per kg) are shown. *Scale bar* = 2 cm.

similar concentration (14 mg per kg per day) was previously shown to chemosensitize effectively human pancreatic carcinoma xenotransplants (Gana-Weisz *et al*, 2002).

In initial toxicologic studies we determined the toxicity of single-dose dacarbazine administration by injecting one dose of 200 mg per kg or 400 mg dacarbazine per kg (i.p.) into SCID mice. Dacarbazine at 400 mg per kg was lethal, killing mice within 3 d of injection. Mice injected with 200 mg dacarbazine per kg showed clear but acceptable signs of toxicity. Combination treatment of 10 or 20 mg FTS per kg (daily for 14 d) and 200 mg dacarbazine per kg (once on day 8 of FTS treatment) yielded high overall toxicity and resulted in only marginal reduction of tumor growth (data not shown). As a dosage of 80 mg dacarbazine per kg administered on 5 consecutive days was previously shown by our group to be well tolerated and highly active in a SCID mouse model (Jansen *et al*, 1998), we adapted this regimen and investigated the anti-tumor effect of 10 mg FTS per kg given daily for 2 wk in combination with 80 mg dacarbazine per kg given on days 1–5 of FTS injection. Mice treated with carrier, carrier plus dacarbazine, single-agent dacarbazine, or saline were used as controls. The shortened FTS treatment period of only 2 wk elicited no response in the 10 mg FTS per kg group compared with the carrier control (**Fig 3*A***). Tumors (n = 8) in both groups showed an equal mean reduction of 20% (1.4 g $\pm$ 0.31 g, for 10 mg FTS per kg and 1.4 g $\pm$ 0.26 g, for the carrier) compared with xenografts in the saline-treated group. Tumors treated with single-agent dacarbazine (n = 8) were reduced by a mean value of 32% (1.2 g $\pm$ 0.3 g), which was exactly the same reduction as that obtained in the tumors treated with a combination of carrier and dacarbazine (1.2 g $\pm$ 0.3 g). Noticeably, the combination of FTS and dacarbazine resulted in a significant reduction in mean tumor growth (n = 8) of 56% (0.7 g $\pm$ 0.17 g; **Fig 3*A***; $p < 0.008$) compared with treatment with saline or single-agent FTS or dacarbazine. These findings demonstrate that in our human melanoma SCID xenotransplantation model the combination of chemotherapy and FTS is clearly superior to treatment with FTS or dacarbazine as single agents.

Differences in actual tumor sizes are seen in photographs of excised xenografts (**Fig 4**). These xenografts are part of the tumor pools (n = 8) used for calculation of the mean tumor weights shown in **Fig 3(*A*)**. Tumors from the FTS/dacarbazine combination treatment group are much smaller than tumors from the single-agent FTS group (10 mg per kg) or the saline control group (**Fig 4**). **Figure 3(*B*)** shows the tumor development during the treatment and observation periods. In xenografts treated with the combination therapy growth was slowed right from the beginning, and this inhibitory potential was maintained beyond the end of treatment and until the end of the observation period. This finding clearly demonstrates the therapeutic value of the Ras-inhibitory drug FTS in combination with the cytotoxic agent dacarbazine over the standard chemotherapy. Mouse body weights at the end of the observation period were similar in all groups (**Fig 2*B***) The saline-treated control mice were the smallest, apparently because of their exceptionally large xenografts; mean tumor weights in this group (1.7 g $\pm$ 0.3 g) represented about 10% of the mean whole body weight (17.5 g $\pm$ 1.2 g). In additional experiments, we assessed the anti-tumorigenic effects of 20 mg FTS per kg plus 80 mg dacarbazine per kg and found that this treatment regimen, owing to higher toxicity and similar treatment response, was not superior to the combination of 10 mg FTS per kg and 80 mg dacarbazine per kg (data not shown).

Previous studies have shown that FTS is well tolerated (Jansen *et al*, 1999; Weisz *et al*, 1999; Gana-Weisz *et al*, 2002). Dacarbazine, however, is known for its potential toxic effects, which include hemopoietic depression causing leukopenia and thrombocytopenia, as well as its hepatic toxicity (Quinio *et al*, 1997; Loo *et al*, 1976; Costanzi, 1976). Analysis of blood cells to evaluate drug toxicities at the end of the observation period revealed no differences between the groups. White and red blood cell counts and platelets were not affected in our model system by FTS or dacarbazine or their combination (data not shown). These data pointed to the low overall toxicity of the drugs 2 wk after the last FTS injection and about 3.5 wk after the last dacarbazine injection. To determine the immediate toxicity of these drug

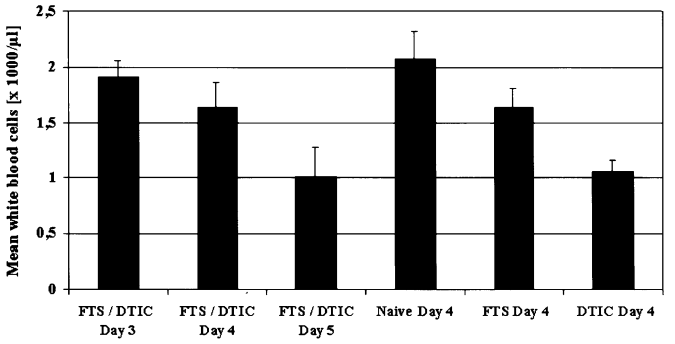

Figure 5. Leukopenia induced by dacarbazine treatment. Tumor-free mice were injected with FTS (20 mg per kg, i.p.), dacarbazine (80 mg per kg, i.p.), a combination of FTS (20 mg per kg) and dacarbazine (80 mg per kg), or were left untreated (naive mice). On the indicated days blood was drawn and white blood cells were counted. Mean cell counts are presented for control groups (naive, FTS, and dacarbazine) and for the combination therapy group (FTS plus dacarbazine). Values are cell counts (mean ± SD, n = 4 per group and per time point).

treatments, we treated tumor-free mice with FTS, dacarbazine, or FTS plus dacarbazine. The mice were killed during the treatment period (see *Materials and Methods* for details) and whole blood and serum samples were analyzed. Erythrocyte numbers were not affected by the treatment used (data not shown), whereas leukocyte numbers decreased during the combination treatment by a mean value of 51% ± 12%, after 5 d. Notably, however, the same reduction was caused by dacarbazine alone (**Fig 5**). The combination of FTS and dacarbazine therefore had no toxic effects on bone marrow depression or production of white or red blood cells beyond those elicited by dacarbazine toxicity. Similar results were obtained for platelets, where no significant changes could be detected in either of the treatment groups (data not shown).

Mouse body weights in all treatment groups were slightly reduced compared with the untreated controls. Mean body weights recorded on the days the mice were killed showed reductions of 19% ± 0.2%, in the group treated with FTS plus dacarbazine and 13% ± 0.1%, in the group treated with dacarbazine alone. The body weights of mice in all other groups were within this range (data not shown).

Side-effects in addition to hemopoietic depression (the most common toxic side-effect of dacarbazine seen in patients) include hepatic toxicity accompanied by hepatic vein thrombosis and hepatocellular necrosis (Loo *et al*, 1976; Quinio *et al*, 1997). Hepatic toxicity has been observed mainly when dacarbazine has been administered concomitantly with other anti-neoplastic drugs, although it has also been reported in some patients treated with dacarbazine alone. Renal dysfunction with raised retention parameters is rather uncommon. We detected no alterations in hepatic, renal, or pancreatic function, suggesting that our experimental animals showed no immediate toxic effects of FTS, dacarbazine, or the combination of FTS and dacarbazine at the concentrations tested (data not shown). Histologic analysis revealed no signs of treatment-related liver pathology (data not shown).

DISCUSSION

As oncogenic Ras is known to play a key role in chemoresistance of human neoplasms (Jansen *et al*, 1997; Satyamoorthy *et al*, 2001), the concept of inhibiting constitutively activated Ras in order to chemosensitize tumors appears to be a rational one. Several ways to inhibit Ras function have been developed. FTI were designed to inhibit the post-translational farnesylation of Ras required for its membrane anchorage and transforming activity (Kohl *et al*, 1993; Cox and Der, 1997). Anti-sense oligonucleotides that selectively prevent the expression of mutated Ras proteins (Aoki *et al*, 1995) reduce the total cellular amount of Ras at the translational level. Restoring catalytic activity to constitutively active Ras proteins (Ahmadian *et al*, 1999), thereby converting them to the inactive GDP-bound form (Scheffzek *et al*, 1998; Ahmadian *et al*, 1999), as well as inhibitors of Ras effector interaction (Herrmann *et al*, 1998), are rather new concepts whose feasibility in Ras-directed cancer therapy has yet to be proven. FTI fail to prevent membrane anchorage of the clinically important N-Ras and K-Ras due to the alternative prenylation of these Ras isoforms via geranyl-geranyl transferase under conditions where the farnesyl transferase is inhibited. Unlike FTI, FTS competes directly with all Ras isoforms for their specific membrane-binding sites, with no obvious specificity for any particular isoform. So far, FTS executed its anti-tumorigenic potential in a number of tumor models (Haklai *et al*, 1998; Jansen *et al*, 1999; Weisz *et al*, 1999; Halaschek-Wiener *et al*, 2000). Although FTS was clearly shown to interact with Ras proteins, we cannot rule out the possibility that inhibition of melanoma cell growth by FTS may be associated with the inhibition not only of active Ras but also of other prenylated proteins; however, there is no evidence to support this rather unlikely notion.

We have previously reported that FTS inhibits cell growth of human melanoma cells, including 607B *in vitro*. Furthermore, suppression of tumor growth by single-agent FTS therapy administered immediately after tumor cell inoculation was demonstrated in melanoma xenotransplantation models (Jansen *et al*, 1999). These results on single-agent FTS activity, obtained in a more artificial model system without established tumors, support the findings reported here. In this study we evaluated the therapeutic potential of FTS on pre-established human melanoma xenografts, a model that more closely mimics the clinical setting. Treatment of subcutaneously grown tumors with 10, 20, or 40 mg FTS per kg daily for 4 wk resulted in a concentration-dependent reduction in mean tumor growth of 31%, 38%, and 45%, respectively. These findings support our previous xenotransplantation studies demonstrating the successful activity of FTS as a single-agent anti-neoplastic drug (Jansen *et al*, 1999; Weisz *et al*, 1999; Gana-Weisz *et al*, 2002).

Based on the considerations outlined above, we nevertheless assumed that the most effective application of FTS in preclinical as well as in clinical studies was likely to be in combination with cytotoxic drugs. Examination of this assumption was the focus of this study. Chemotherapeutic agents are the drugs most commonly used in clinical cancer therapy. Success rates vary greatly, however, depending on the tumor type and the stage of disease progression. Newer strategies of cancer treatment often include combination chemotherapies in which specific anti-tumor compounds, with known mechanisms of action, are combined with traditional chemotherapeutic agents (Cohen and Falkson, 1998; Nathan and Mastrangelo, 1998; Huncharek *et al*, 2001). In a recent study, FTS was found to be a possible partner in conventional chemotherapy regimens leading to chemosensitization of human colon and pancreas cancer cells *in vitro* and (in the latter case) also *in vivo* (Gana-Weisz *et al*, 2002). The basic concept is that oncogenic Ras leads to increased chemoresistance and higher survival rates of cancer cells by streamlining the survival signaling pathways and short-circuiting the natural apoptotic machinery (Serrone *et al*, 2000; Satyamoorthy *et al*, 2001). Especially in human malignant melanoma activated N-Ras was shown to confer chemoresistance, indicated by the finding that this oncogenic Ras isoform decreases chemotherapy-induced apoptosis in melanoma xenotransplantation models (Jansen *et al*, 1997). In addition, FTS was recently shown to block human melanoma growth through a combination of cytostatic and pro-apoptotic effects *in vitro* (Smalley and Eisen, 2002). We show here that combination therapy of FTS and dacarbazine is significantly superior to single-agent use of either of these drugs (see **Figs 3*A* and 4**). Our initial experiments showed that FTS at 10 mg per kg had only a small effect on tumor growth, even when given for 4 wk (see **Fig 1*A*,*B***). In a second set of experiments we combined FTS and dacarbazine over shorter treatment periods, and found that

FTS at 10 mg per kg had no anti-tumor effects. Dacarbazine alone or in combination with the carrier showed a marginal response, whereas in comparison, the combination therapy with FTS and dacarbazine resulted in significant tumor growth reduction ($p < 0.008$) relative to either FTS, dacarbazine or saline (see **Fig 3*A***). Inhibition of tumor growth persisted even during the observation period after FTS treatment was stopped (see **Fig 3*B***).

Standard treatment regimens for human melanoma include single-agent dacarbazine as well as dacarbazine-based combination chemotherapies (Serrone *et al*, 2000; Huncharek *et al*, 2001). Clinically, dacarbazine is administered differently in European and American treatment regimens. In the European treatment scheme dacarbazine is administered five times at 200 mg per m^2, whereas the American regimen is 1000 mg per m^2 given once. We initially followed the American pattern and applied single doses of 200 or 400 mg dacarbazine per kg in our experiments. The dosage of 400 mg per kg turned out to be lethal, whereas 200 mg per kg induced toxic side-effects and produced only a small increase in the treatment response when administered in combination with FTS. Previously, however, our group reported that 80 mg dacarbazine per kg given on 5 consecutive days is well tolerated and highly active in SCID mice (Jansen *et al*, 1998). This dacarbazine treatment scheme was also found to be suitable in our FTS combination studies, in terms of both toxicity and anti-tumor activity.

Based on our present results and recently described findings (Jansen *et al*, 1999; Weisz *et al*, 1999; Halaschek-Wiener *et al*, 2000; Gana-Weisz *et al*, 2002), we suggest that FTS has the potential ability to interfere with mutated Ras proteins and Ras signal transduction in a number of different cancer types, including colon and pancreatic cancers and human melanoma. The Ras inhibitor FTS clearly enhances the cytotoxic effect of dacarbazine in our SCID mouse model and, most notably, it does so without contributing further to the toxicity.

Interestingly, a recent study reported a high incidence (66%) of a somatic mis-sense mutation in the B-Raf gene of human melanoma, where the most frequent V599E mutation is an activating mutation (Davies *et al*, 2002). As B-Raf acts downstream of Ras, this could indicate that Ras inhibitors may not be considered as suitable candidates for melanoma therapy. Based on current knowledge of Ras biology and on the data presented on the activated B-Raf mutants, however, we believe that Ras inhibitors are potentially promising therapeutic agents for human melanoma. First, Ras is pleiotropic, activating many downstream targets other than B-Raf, some of which (e.g., PI3-K and Ral-GEF) are critical for maintenance of the transformed phenotype of human tumor cells. Second, the activity of activated B-Raf mutants is also partially Ras dependent, and is therefore enhanced by activated Ras (Davies *et al*, 2002). Third, although human cell lines containing V599E B-Raf mutations do not require Ras for proliferation, it is not yet clear whether or not they require Ras for survival, invasion, and migration.

The use of specific inhibitors of key cellular functions, in combination with so-called "dirty" conventional drugs with a myriad of mechanisms of action, might be the route to take in our quest for improving treatment outcome. Rational combinatorial strategies have led to a paradigm shift in human immunodeficiency virus therapy (Clumeck and De, 2000; Telenti and Paolo-Rizzardi, 2000), and it is reasonable to speculate that similar strategies may prove successful in the treatment of resistant malignancies. FTS appears well positioned to become a key tool in the fight against malignancies where mutated Ras genes and Ras signal transduction play an important part in oncogenesis, disease progression, and treatment resistance.

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